United States
Environmental Protection
Agency

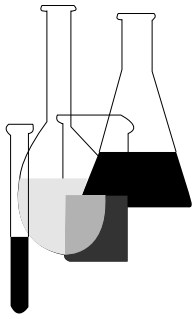
Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712–C–96–156
April 1996

EPA

Ecological Effects Test Guidelines

OPPTS 850.4400 Aquatic Plant Toxicity Test Using *Lemna* spp., Tiers I and II



"Public Draft"

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

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OPPTS 850.4400 Aquatic plant toxicity test using *Lemna* spp., tiers I and II.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline are 40 CFR 797.1160 *Lemna* Acute Toxicity Test and OPP 122–2 Growth and Reproduction of Aquatic Plants (Tier I) and 123–2 Growth and Reproduction of Aquatic Plants (Tier 2) (Pesticide Assessment Guidelines, Subdivision J—Hazard Evaluation; Nontarget Plants) EPA report 540/09-82-020, 1982.

(b) **Purpose.** This guideline prescribes test procedures and conditions using the freshwater aquatic plants *Lemna gibba* and *L. minor* to develop data on the phytotoxicity of chemicals (see paragraphs (g)(1), (g)(2), and (g)(3) of this guideline). The Environmental Protection Agency will use data from these tests in assessing the hazard of a chemical to the environment.

(c) **Definitions.** The definitions in section 3 of TSCA and 40 CFR part 792—Good Laboratory Practice Standards are applicable to this guideline. The following definitions also apply:

Axenic means a culture of *Lemna* fronds free from other organisms.

Colony means an aggregate of mother and daughter fronds attached to each other.

ECX means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

Frond means a single *Lemna* ‘‘leaf-like’’ structure.

Frond mortality means dead fronds which may be identified by a total discoloration (yellow, white, black, or clear) of the entire frond.

LOEC is the lowest test concentration of a material used in this test that has an adverse effect.

MATC is the hypothetical toxic threshold concentration lying in a range bounded by the NOEC and LOEC. GmMATC is the geometric mean of these values.

NOEC is the highest test concentration of a material used in this test that does not have an adverse effect.

Static-renewal test means a test method in which the test solution is periodically replaced at specific intervals during the test.

(d) **Test procedures**—(1) **Summary of the test.** (i) In preparation for the test, containers are filled with appropriate volumes of nutrient medium and/or the test solutions. The test is started by introducing *Lemna* fronds into each of the containers. Nutrient medium and test solutions may need to be replaced on day 3 or 5, or as needed to prevent nutrient limitation or depletion of the test chemical. Periodic renewal (static-renewal) will help to maintain constant exposure concentrations of the test chemical over the test period for compounds that are unstable in water. In a 14-day test, renewal may be necessary every 3 to 5 days.

(ii) Colonies should be inspected for changes in frond number and appearance at the beginning of the exposure period (day 0), on days 3, 5, and at the end of the exposure period (day 7). On day 7, the total number of living and/or dead fronds are counted. Any frond which is visible as a bud when viewed under a hand lens or dissecting microscope should be counted. Concentration response curves are plotted for total frond number, growth rate (as number of fronds per day) and mortality (percentage of dead fronds to total number of fronds). EC5s, EC50s, and EC90s are determined from the curves.

(2) **Range-finding test.** (i) A range-finding test should be conducted to establish if definitive testing is necessary and to determine test solution concentrations for the definitive test. Water solubility of the test chemical (as well as other physical chemical characteristics, e.g. volatility) should be determined before definitive testing. A validated analytical method should also be developed prior to any toxicity testing.

(ii) The recommended procedure is to expose *Lemna* to a chemical concentration series (e.g., 0.1, 1.0, 10, 100, and 1,000 mg/L plus controls. For pesticide testing under FIFRA, one concentration equivalent to the maximum recommended label rate is sufficient (Tier I, 40 CFR part 158). A minimum of three replicates of three to five plants consisting of three to four fronds each should be added to each test chamber. Plants of similar size should be selected, and the number of plants and number of fronds should be identical or as near identical as possible in each test chamber. A total of at least 12, but no more than 16 fronds, per test chamber are recommended (e.g. three 4–frond plants and one 3–frond plant could be used, for a total of 15 fronds). Plants are exposed to equal volumes of each chemical concentration for a period of 7 days. It has been shown that sufficient numbers of plants are produced within 7 days to provide adequate results (see paragraph (g)(2) of this guideline).

(iii) The lowest chemical concentration in a test series, exclusive of controls, should be the lowest concentration which can be analytically quantified; except for pesticide testing under FIFRA. The highest concentration should be at least 1,000 mg/L; except for pesticide testing under FIFRA. Replicates are not needed and nominal concentrations of the chemical are acceptable for range-finding. If testing a pesticide under FIFRA

at the maximum labeled dosage, a minimum of three replicates is required. If the calculated 50 percent inhibition value is greater than 1,000 mg/L (greater than the maximum label rate if a pesticide is tested under FIFRA) or is less than the analytical detection limit, definitive testing is not necessary. However, replicates and measured concentrations of the appropriate dose are needed to substantiate this result.

(3) **Definitive test.** (i) The purpose of the definitive test is to determine the EC5s, EC50s, EC90s, LOECs, and NOECs for *Lemna* growth based on total frond number, growth rate, and/or frond mortality with a minimum amount of testing beyond the range-finding test. Other end-ponts (optional) include dry weight and chlorophyll and pheophytin pigment analyses, under paragraphs (g)(2) and (g)(3) of this guideline.

(ii) At least five concentrations of chemical, exclusive of controls, should be used in the definitive test and chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g. 2, 4, 8, 16, 32, 64 mg/L). The concentration range should be selected to define the concentration response curve between the EC5 and EC90. For each concentration and control at least three replicate containers should be used, each containing 150 mL of test solution, or enough test solution to result in a volume-to-vessel size ratio of 2:5, and three to five plants consisting of three to four fronds each should be used. See paragraph (d)(2)(i) of this guideline for a discussion on plant size and frond numbers to be used in the definitive test. Fewer replicates, each containing a greater number of colonies, may be used; however, test containers and solution volumes will have to be adjusted accordingly. The range of chemical concentrations tested should result in the highest concentration affecting at least 90 percent of the fronds and the lowest concentration affecting no more than 5 percent of the fronds compared with the controls or the selected test concentrations should bracket the expected EC50 value.

(iii) Every test should include controls consisting of the same nutrient medium, number of fronds, environmental conditions, and procedures as the test containers except that none of the chemical is added. If a solvent or carrier is used to dissolve or suspend the test chemical, additional controls containing the solvent or carrier should also be included in the test to determine any effect of the solvent or carrier on the plants. The upper limit of carrier volume is 0.5mL/L and the same amount of carrier should be added to each test concentration.

(iv) Positive controls using zinc chloride as a reference chemical should be run periodically. The purpose of a running a positive control with a reference chemical is to determine if the test *Lemna* fronds are responding to a known chemical in the expected manner. If the fronds respond to subsequent reference chemical tests consistently, it is assumed that the *Lemna* will respond to other chemicals consistently. Changes in *Lemna* response caused by factors such as poor nutrition, genetic drift,

and contaminants, may not be detected by negative controls, yet may still influence test results. A minimum of three concentrations of the reference chemical are run at or near the expected median effect level.

(v) The colonies should be transferred to test solution on days 3 and 5. No more than 20 percent of the test substance should be lost by volatilization (nor by processes such as hydrolysis, photolysis, etc.) between replacements. Flow-through procedures may be necessary for volatile substances.The colonies may have to be transferred more frequently for highly volatile test substances in order to maintain 80 percent of the initial test substance concentration. Transfer should be done in a clean, draft-free area as quickly as possible to minimize contamination of the colonies.

(vi) Observations of frond numbers and appearance should be made of the colonies on day 0, 3, 5, and 7. A dissecting microscope will facilitate observations.

(vii) Concentration response curves should be plotted. These curves can provide the basis for determining the EC5s, EC50s, and EC90s for total frond number, growth rate, and plant necrosis.

(viii) Any change in frond development or appearance such as increase in number (a frond is counted regardless of size as long as it is visible adjacent to the parent frond), decrease in size, necrosis, chlorosis, etc., should be reported. Any additional observations such as sedimentation of test solution, sinking of fronds, or other abnormalities should also be recorded.

(ix) Other end-points that can be assessed in this test and that have been shown to be indicative of growth inhibition are chlorophyll values (chlorophyll *a*, *b*, *c*, and pheophytin *a* pigments) and biomass (dry weight at 60 °C) at the end of the test (refer to paragraphs (g)(2) and (g)(3) of this guideline).

(x) A randomized complete block design is recommended for the definitive test with blocks delineated within the test chamber. If, for any reason, blocking is not feasible, total randomization within chambers is suggested.

(4) **Analytical measurements**—(i) **Chemical.** Stock solutions or growth media should be prepared just prior to use and diluted with water of high quality such as glass-distilled, deionized water, or ASTM Type I to obtain the test solutions. Volume increases should be limited to prevent dilution of the nutrients. Standard analytical methods, if available, should be used to establish concentrations of these solutions and should be validated before beginning the test. An analytical method is not acceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference. The pH of the test solutions should also be measured prior to and after use. Chlorophyll

and pheophytin pigment analyses should be based on accepted procedures under paragraphs (g)(2) and (g)(3) of this guideline.

(ii) **Numerical.** The number of fronds is counted at the end of the definitive test. Means and standard deviations are calculated and plotted for each treatment and control. Appropriate statistical analyses are used to provide a goodness-of-fit determination for the concentration response curves.

(e) **Test conditions**—(1) **Test species.** The test species to be used in these tests are *L. gibba* G3 and *L. minor*. Axenic cultures may be obtained from laboratory cultures or commercial sources. A stock culture grown from a single isolated plant should be used to inoculate all the flasks used in a given test.

(2) **Stock cultures.** Axenic stock cultures should be grown in the aquariums for 2 weeks (with necessary transfers) prior to being used in a test. Plants used in a test should be randomly selected from the culturing tank. Inocula should be taken from cultures which are less than 2 weeks old.

(3) **Facilities**—(i) **Apparatus.** (A) A controlled environment growth chamber or an enclosed area capable of maintaining the specified number of test chambers and test parameters (see paragraph (e)(4) of this guideline) is needed.

(B) Laboratory facilities for the mixing and diluting of nutrient medium and a source of distilled or deionized water are needed. An autoclave (for sterilizing glassware and media) and a sterile transfer hood (for maintenance of an axenic *Lemna* culture) are also necessary. Disposal facilities should be adequate to accommodate spent test solutions and plant materials as well as any bench covering, lab clothing, or other contaminated materials.

(ii) **Containers and support media.** Test containers may be 250-mL glass beakers or Erlenmeyer flasks, large enough to hold 150 mL of test solution and the *Lemna* colonies without crowding for the duration of the test. All containers should be of the same type and size. Even though at least three replicates are used, larger containers may sometimes be necessary to hold additional colonies and test solution volume. The ratio of test solution volume to container volume should not exceed 2:5. For each test concentration and control the same number of replicates should be used.

(iii) **Cleaning and sterilization.** All glassware and equipment should be cleaned following good laboratory practice. The Nytex screen or inoculating loops used for transferring the *Lemna* should be disposed of after use or thoroughly cleaned and sterilized before reuse.

(iv) **Nutrient media.** M-Hoagland's or 20X-AAP nutrient media are recommended for maintaining *Lemna* cultures and for use as the diluent in the preparation of various concentrations of test solutions under paragraph (g)(1) of this guideline. Water of high quality (ASTM Type I for example) can be used to make the nutrient medium. The medium should be prepared prior to each transfer of *Lemna* cultures and for the preparation of new test solutions during the course of a test. If prepared in advance it should be refrigerated. The pH of the medium should be adjusted to between 4.8 to 5.2 by the addition of 0.1N or 1 N NaOH for culture maintenance prior to addition of the test chemical for M-Hoagland's medium. If 20X-AAP medium is used, the pH should be adjusted to 7.5 ± 0.1 with 0.1 N NaOH or HCl (see paragraph (g)(1) of this guideline). Nutrient media should not contain organic metabolites such as sucrose. Chelating agents, such as EDTA, are present in the 20X-AAP medium to ensure that trace nutrients will be available to the *Lemna* fronds. M-Hoagland's medium (which contains no EDTA) should be used for test solution preparation if it suspected that the chelator will interact with the test chemical.

(v) **Carriers.** Stock solutions of substances of low aqueous solubility may be prepared by use of organic solvents, emulsifiers, or dispersants of low phytotoxicity to plants. When a solvent or carrier is used, a second set of controls should contain the same concentration of the solvent or carrier as that used in the highest concentration of the test substance. The concentration of the solvent or carrier should not exceed 0.5 mL/L and the same amount of carrier should be added to each test concentration (see paragraph (g)(1) of this guideline).

(4) **Test parameters.** Environmental conditions should be maintained as specified below:

(i) Temperature at 25 ± 2 °C.

(ii) The pH of the nutrient medium between 4.8 and 5.2 for M-Hoagland's medium, 7.5 ± 0.1 for 20X-AAP medium. Test solution pH may vary from the nutrient medium after the addition of the test chemical and/or carrier (if used). Any such changes should be recorded but not adjusted.

(iii) Continuous warm-white fluorescent lighting should be used to provide a light intensity in the range of 4,200 and 6,700 lx (394 and 620 fc), as measured adjacent to each test chamber at the surface of the test solution. The light intensity at each position in the incubation area should be measured and should not differ by more than 15 percent from the selected light intensity.

(f) **Reporting.** Reporting requirements of 40 CFR part 792—Good Laboratory Practice Standards apply to this guideline. The following data should also be reported.

(1) Source of *Lemna* and taxonomic verification.

(2) Description of test chambers, type of lights, size of beakers or flasks used, number of concentrations and replicates per concentration, number of colonies per replicate, solution volumes, physical parameters of growth chambers (e.g. temperature, and light intensity).

(3) The pH and concentration of the test chemical in the test solutions prior to use and discarding on day 3, 5, and 7.

(4) Number of fronds per test concentration and control at the end of the test, the percent inhibition and/or stimulation of growth rate, and percent frond mortality for each test concentration compared to controls.

(5) If the range-finding test showed that the highest concentration of the chemical tested (not less than 1,000 mg/L or the maximum pesticide label application rate) had no effect on *Lemna*, report the results and measured concentrations and a statement that the chemical is not phytotoxic at concentrations less than 1,000 mg/L.

(6) If the range-finding test showed greater than a 50 percent effect with a test concentration below the analytical detection limit, report the results and a statement that the chemical is phytotoxic below the analytical detection limit.

(7) Charts of growth in the no-treatment and solvent controls, on each counting day and at the end of the test, for each toxicity test.

(8) Means and standard deviations for frond number, growth rate, and percent frond mortality in each test concentration. In addition, concentration response curve(s) with 95 percent confidence limits delineated, goodness-of-fit determination, and EC5s, EC50s, EC90s, LOECs, and NOECs identified (see paragraph (g)(4) of this guideline).

(9) Methods and data records from chemical and numerical analyses including validation methods and quality assurance procedures.

(g) **References.** The following references should be consulted for additional background material on this test guideline.

(1) American Society for Testing and Materials. ASTM E1415-91. Standard guide for conducting static toxicity tests with *Lemna gibba* G3. In 1991 Annual Book of ASTM Standards, vol. 11.04: Pesticides; resource recovery; hazardous substances and oil spill response; waste disposal; biological effects, pp 1137–1146 (1991).

(2) Cowgill, U.M. and D.P. Milazzo, The culturing and testing of two species of duckweed, pp 379–391 in U.M. Cowgill and L.R. Williams (eds.). *Aquatic Toxicology and Hazard Assessment*, 12th volume, ASTM STP 1027 ASTM, Philadelphia, PA (1989).

(3) Taraldsen, J.E. and T.J. Norberg-King, New method for determining effluent toxicity using duckweed (*Lemna minor*). *Environmental Toxicology and Chemistry* 9:761–767 (1990).

(4) Bruce, R.D. and D.J. Versteeg. A statistical procedure for modeling continuous toxicity data. *Environmental Toxicology and Chemistry* 11:1485–1494 (1992).